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Influence of explant source, plant growth regulators and culture environment on culture initiation and establishment of *Quercus robur* L. *in vitro*

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Abstract

Suitable cytokinin supplements and culture environments have been determined for the initiation and establishment of shoot cultures of *Quercus robur* seedling tissue. Initiation of axillary shoot development from nodal explants required culture medium supplemented with BA (6-benzylamminopurine). The greatest numbers of stem segments for culture proliferation were obtained using 1.0 mg l^{-1} BA after 56 d culture. The frequency of shoot development and subsequent formation of multiple shoots at initiation was influenced by the position of the nodal explant in the seedling shoot, incubation temperature and daylength. Explants from basal and apical regions, which contained multiple axillary buds, produced the lowest frequencies of axillary shoot development and multiple shoot formation, many remained quiescent. Axillary shoot development was greatest in single nodal explants excised from the mid-stem positions, elongated regions of the shoot where nodes were formerly associated with a leaf. Higher temperatures stimulated shoot formation with greater numbers of stem segments for culture multiplication being obtained from nodal explants incubated at 25 °C. Axillary shoot development was promoted in nodal explants maintained under daylengths of 16 h or more. Stem segments cut from axillary shoots which developed from nodal explants were used to establish shoot multiplication cultures on medium supplemented with 0.4 mg l^{-1} BA. Shoot formation from stem segments was greater at higher incubation temperatures of 25 °C and 30 °C. Multiplication coefficients for stem segments increased after one subculture.

Key words: *Quercus robur*, oak, micropropagation, cytokinin, temperature, daylength, rest, quiescence.

Introduction

Newly isolated explants from hardwood tree species often remain quiescent for several weeks or months, and McCown and McCown (1987) have also observed this phenomenon in tissues excised from newly formed shoots in culture. The problem of quiescence is greater in species which naturally exhibit episodic growth, such as *Quercus*, *Fagus*, *Castanea*, and *Carya*, and represents a major restriction to their micropropagation (McCown and McCown, 1987). In such species, shoot growth is dominated by internal controls, with each phase of growth occurring from preformed buds, once or twice over the growing season. Such rhythmic growth is also evident in culture (Favre and Juncker, 1987).

The initiation of explants of species with episodic growth often appears to be successful. Initial growth in culture, which can be very rapid, represents the expression of buds formed on the 'mother' plant. Rates of *in vitro* axillary shoot development of oak are influenced by the developmental stage of the source plant, being slower from explants excised from elongating shoots compared to those from explants taken when internode elongation is complete (Volkaert *et al.*, 1990). Clearly, the initial success of a culture is determined before material has been isolated but, as Evers *et al.* (1988) report, methods for the micropropagation of *Quercus* species often lack information on the condition of the starting material.

Once buds on the isolated explant have fully elongated, the rapid growth phase usually ends. Transferring shoot

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Table 1. *The effect of BA on the initiation of axillary shoot growth in nodal explants of* Q. robur

Values are based on 20 explants per treatment.

Concentration of BA (mg l^{-1})	Number of contaminated explants	Number of productive explants forming a shoot	Mean number of shoots per productive explant	Mean length of tallest shoot (mm)	Mean number of segments per productive explant	Multiplication coefficient (mean number of segments per explant)
28 d						
0	2	8	1.0	9.6	1 3	0.6
0 1	2	15	2.2	18.4	3.6	3.0
0.2	2	14	1.4	20.2	2.6	2.0
0.4	2	13	2.4	17.3	3.5	2.5
0.6	1	16	2.6	17.9	3.7	3 1
0.8	2	11	2.3	15.5	3.1	1.9
1.0	4	11	2.8	18.6	4.3	2.9
Significance						
χ^2(df)		9.6(6)				
sed(df)			0.48(81)**	3.2(81)	0.89(81)	0.67(118)**
Lin(SE)[a]			1.3(0.37)***	0.39(0.42)	1.6(0.61)***	—
56 d						
0	2	5	1.0	10 4	1.0	0.3
0.1	2	14	1.9	11.9	2.3	1.8
0.2	2	13	1.5	13.1	1.9	1.4
0.4	2	15	2.7	12.7	3.5	2.9
0.6	2	11	2.6	14.7	3.6	2.2
0.8	2	9	2.7	16.0	3.3	1.7
1.0	4	12	3.0	15.8	4.8	3.6
Significance						
χ^2(df)		17.6(6)**				
sed(df)			0.58(72)***	3.31(72)	1.01(72)**	0 66(117)***
Lin(SE)[a]			1.7(0.37)***	0.9(0.37)*	2.9(0.67)***	—

Asterisks denote significant differences; $*P<0.05$, $**P<0.01$, $***P<0.001$.

[a]Lin estimated value for slope of linear trend. No values are presented where deviations from the linear trend are significant.

SE = standard error.

sections to new media does not guarantee continued growth and a protracted period of quiescence can ensue. Quiescence or rest has been reported to occur in oak explants after subculture, the problem is greater when older source plants are used but also arises in embryo derived material and can lead to loss of the culture (Evers *et al.*, 1988). Such responses suggest that some feature of the chemical or physical environment is limiting. Following subculture, subsequent growth is dependent on meristems generated in culture, such that the culture environment now has the dominant effect. This represents the establishment phase of the culture where growth is often unpredictable (McCown and McCown, 1987). Cultural conditions, such as medium composition, use of growth regulators, light and temperature regimes, can substantially modify the growth of explants.

The effects of factors such as mineral nutrients (Chalupa, 1984*a*; Vieitez *et al.*, 1985; Civinová and Sladký, 1987; McCown and Sellmer, 1987) and growth regulators (Chalupa, 1984*b*; Pevalek-Kozlina and Jeleska, 1986; Minocha, 1987; Chalupa, 1988) on culture establishment of hardwood trees, including oak, has been addressed. The influence of the culture environment on the establishment of hardwood tree explants has not been widely investigated and reported incubation conditions vary widely. Oak cultures have been maintained under constant temperatures ranging from 25–28 °C (Favre and Juncker, 1987; Evers *et al.*, 1988; Chalupa, 1988) and under fluctuating regimes, employing higher day temperatures (24–30 °C) and cooler nights (18–20 °C) (Chalupa, 1984*b*; Vieitez *et al.*, 1985; San-José *et al.*, 1988). Similarly, both constant irradiance periods (Volkaert *et al.*, 1990) and 16/8 h light/dark cycles (Favre and Juncker, 1987; Evers *et al.*, 1988; Vieitez *et al.*, 1985) have been used. The significance of these various regimes on *in vitro* axillary shoot development is not explained. The importance of optimizing the culture environment for oak is revealed by the influence that environmental parameters exert on episodic shoot growth *in vivo*. High temperatures and high rainfall are known to favour growth (Pilcher and Gray, 1982) and repeated flushing of oak shoots occurs under long days (Longman and Coutts, 1974; Harmer, 1989). This paper reports the influence of light and temperature regimes on the initiation and establishment of oak cultures against the background of determining the reliability of published protocols.

Table 2. *Formation of axillary shoots from nodal explants excised from different seedling shoots each possessing 14 lateral buds*

Shoot (provenance/rep. no.)	Nodal explants with shoots at each position														Total number of explants with shoots
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
NL3/1	–	–	–	–	–	–	+	+	+	–	–	–	–	–	3
NL3/2	+	+	–	+	–	+	+	+	+	+	+	–	+	–	10
NL3/3	–	–	–	+	+	+	+	+	+	+	+	–	–	–	9
NL3/4	+	+	+	+	+	+	+	+	+	+	+	–	–	–	11
NL3/5	+	+	–	+	+	+	+	+	+	+	+	–	–	+	10
NL3 total															**43**
NL5/1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	13
NL5/2	–	–	–	–	–	–	–	–	–	+	+	–	–	–	2
NL5/3	+	+	–	–	+	+	+	+	+	+	+	+	–	–	10
NL5/4	–	+	+	+	+	+	–	+	+	+	+	+	+	+	12
NL5/5	–	–	+	–	–	–	+	+	+	+	+	+	–	–	7
NL5 total															**44**
Proportion of leafless explants (%)	100	100	100	100	90	80	60	20	0	0	0	0	0	0	
Total number of productive explants forming a shoot	5	6	4	6	5	7	8	9	9	9	9	5	2	3	

Significance

Change in deviance for provenances: $\chi^2 = 0.03$, df = 1, ns

Change in deviance for shoots: $\chi^2 = 36.5$, df = 9, $P < 0.001$

Change in deviance for positions: $\chi^2 = 33\ 3$, df = 13, $P < 0.01$

Materials and methods

Plant material and growth conditions

Quercus robur seeds of two Dutch provenances (NL3 and NL5) were purchased from Cambridge Direct Seeding (Cambridge, UK) and stored at 2 °C in cloth bags containing moist peat with 1% Benlate until required. Acorns were germinated in a peat based compost containing moss peat (300 l), magnesium limestone (606 g), superphosphate (404 g), potassium nitrate (180 g), WM 255 frit trace elements (72 g) in 250 ml Rootrainers (Ronash Ltd, Roxburghshire). Seedlings of both provenances were raised for each experiment between March and October in a heated glasshouse with venting above 15 °C and a minimum daylength of 16 h maintained by the use of tungsten bulbs (60 W) at 0.5 m centres 50 cm above the plants. Plants were watered daily and treated with pesticides when required; dimilin for sciarid fly, dicofol or flucythrinate for red spider mite, deltmethrin for aphids, and Systhane (myclobutanil) or Nimrod T (bupirimate and triforine) for mildew.

Explant source and explantation timing

For each experiment, seedlings which had reached the same stage of development were selected. The *Quercus* Morphological Index (QMI) described by Hanson *et al.* (1986) and developmental stages defined by Volkaert *et al.* (1990) were used to identify seedlings which had completed their first flush of growth and reached the flush lag (lag) stage. Seedlings at this stage were identified as those which had completed internode elongation and whose middle leaves were fully expanded; most of the upper leaves may still be elongating at this stage (Hanson *et al.*, 1986). Expanded leaves of selected seedlings were green, but had yet to take on the dark green appearance which is typical later in the flush lag stage. From the selected seedlings nodal explants, 15 mm in length and possessing a single lateral bud, were excised from the region of greatest internode elongation. Leaves associated with nodes were removed before surface sterilization. Unless otherwise specified, explants were not excised from basal or apical regions of shoots where internode elongation is restricted and prevents excision of

Table 3. *The influence of explant position on multiple shoot formation in nodal cultures of* Q. robur

Values are based on 10 explants for each position.

	Position of node														Significance
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Number of explants with multiple shoots	2	4	2	3	4	4	4	5	5	5	4	2	0	0	χ^2(df) = 23.2(13)*
Mean number of additional shoots per explant	0.2	0.8	1.2	1.1	1.0	1.4	2.1	1 6	1.1	1.4	2.2	0.5	0	0	sed(df) = 0.81(126)ns

Asterisks denote significant differences with $*P < 0.05$. ns = differences not significant.

explants possessing a single axillary bud. For each experiment equal numbers of explants were excised from selected seedlings of each provenance and randomly placed into experimental treatments.

Culture initiation, media and conditions

Explants were surface-sterilized either in 20% sodium hypochlorite for 5 min or by successive immersions for 20 min in 10% calcium hypochlorite and 0.3% mercuric chloride followed by three successive washes in sterile purified (reverse osmosis) water. Explants were placed individually into 100 ml culture jars containing 20 ml basal medium which consisted of the macronutrients of Greshoff and Doy (1972), the micronutrients and Fe-EDTA listed by Murashige and Skoog (1962), the vitamins listed by Vieitez *et al.* (1985), 30 g l^{-1} sucrose and 6 g l^{-1} agar. Basal medium was adjusted to pH 5.2 before autoclaving at 121 °C for 15 min. Cultures were incubated at 22 °C under a 16/8 h (day/night) daylength cycle with an irradiance of 60 μmol m^{-2} s^{-1} at the culture level provided by warm white fluorescent tubes (Phillips colour 29, 65–85 W), unless otherwise specified.

Influence of cytokinin: To determine the optimal cytokinin treatment for culture initiation, 20 nodal explants were incubated on basal medium, supplemented with BA (6-benzylaminopurine) at the following concentrations 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg l^{-1}. After 28 d, newly developed axillary shoots were excised leaving a basal portion (2–4 mm) of stem on the original nodes which were then returned to fresh medium. Growth responses were recorded after a further 28 d.

Influence of explant source: To determine whether the position of the explant on the source shoot influenced the development of lateral buds during culture initiation, explants were taken from ten selected oak seedlings, each of which had reached the flush lag stage and produced 14 lateral buds. Nodal explants were excised from each shoot for each lateral bud, these being numbered separately and acropetally (1–14). Explants excised from the apical portion of shoots (positions 9–14) possessed large buds which had formerly been associated with a leaf. Basal explants possessed smaller leafless buds (positions 1–5). The type of bud associated with explants excised from intermediate positions was variable. Explants designated as positions 5–10 were excised from regions of the shoot with greater internode elongation. Because internodal expansion is restricted at the base and apex, explants containing two or three lateral buds were excised for these positions. Explants were incubated on basal medium supplemented with 1.0 mg l^{-1} BA and non-destructive measurements of shoot development and shoot height were made every 2 or 3 d. Explants were not subcultured and the experiment was ended after 48 d.

Influence of culture environment: To determine whether the incubation temperature or daylength influenced culture initiation, a series of experiments was conducted in which nodal explants were incubated on basal medium supplemented with 1.0 mg l^{-1} BA under an irradiance of 35 μmol m^{-2} s^{-1} provided by Phillips colour 32, 15 W Deluxe warm white fluorescent tubes and the following conditions: (a) 15, 20, 25, and 30 °C, with a 16/8 h (day/night) daylength cycle; (b) 8, 12, 16, and 24 h of light in a 24 h period and maintained at 22 ± 2 °C; (c) daylengths of 12 and 16 h light in a 24 h cycle at 15 °C and 20 °C. Thirty explants were used in each treatment.

Culture establishment

Axillary shoots formed by nodal explants in culture initiation experiments were excised and cut transversely into 8 mm stem segments containing at least one lateral meristem. Segments were placed individually into 100 ml culture jars containing 20 ml of basal medium; basal medium and incubation conditions are as described for culture initiation. Segments cut from axillary shoots formed in initiation experiments were placed randomly across the treatments used in the following establishment experiments.

Influence of cytokinin. To determine the influence of cytokinin on culture establishment, segments were placed on basal medium supplemented with 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg l^{-1} BA. Thirty segments were used for each BA treatment and the growth of explants was recorded after 28 d.

Influence of incubation temperature: To determine whether incubation temperature influenced culture establishment, segments were placed on basal medium supplemented with 0.4 mg l^{-1} BA and incubated at either 20, 25 or 30 °C, under an irradiance of 35 μmol m^{-2} s^{-1} provided by Phillips colour 32, 15 W Deluxe warm white fluorescent tubes with a 16/8 h (day/night) daylength cycle. Twenty segments were used in each temperature treatment. After 28 d, new shoots > 5 mm in length were harvested and segments were transferred to fresh medium. The experiment was terminated after 56 d.

Growth parameters and analysis

At each subculture the following observations were made: the number of explants with axillary shoots (productive explants); the number of axillary shoots per explant; the height of the tallest shoot; the number of segments generated per active explant, obtained by dividing newly formed axillary shoots into 8 mm nodal and apical segments; and the multiplication coefficient, defined as the proportion of explants forming axillary shoots (productive explants) × the mean number of new 8 mm segments per productive explant and is equivalent to the mean number of new segments per explant. For the analysis of data, the responses of productive explants were scored into categories according to the number of shoots or segments produced. The mean numbers of shoots and segments excised from productive explants are presented.

The effects of treatments on the number of productive explants were analysed by fitting a generalized linear model with either a binomial or poisson distribution of errors, and analyses of deviance. Where the results suggested that differences could be explained by an association of treatment effects (e.g. no BA versus inclusion of BA) one degree of freedom contrasts were estimated to examine these hypotheses. Differences between treatments for the remaining growth parameters were compared by analysis of variance. Tests were conducted at the $P = 0.05$ significance level. For experiments where the use of different concentrations of BA was employed and for positional effects, the data was tested for linear trends. Estimates of the slope of linear trends are quoted where deviations from the fitted line were not significant.

Results

Culture initiation

Influence of BA: inclusion of BA in the basal medium increased the number of explants forming axillary shoots

(Table 1). Differences between treatments proved not to be significant after 28 d, although a comparison of all BA treatments inclusive versus no BA revealed a nominally significant effect ($\chi^2(df)=6.1(1)$, $P<0.05$). Transfer of explants to fresh medium did not increase the number of explants producing axillary shoots on medium lacking BA and most were quiescent. Differences in the number of explants producing axillary shoots between treatments were significant after 56 d ($P<0.01$), after the comparison of BA with no BA ($\chi^2(df)=11.4(1)$, $P<0.001$) had been made the residual treatment differences were not significant. Following transfer to fresh medium the number of explants forming axillary shoots declined in several treatments. This decline was due to necrosis of the explant following excision of shoots formed in the previous subculture.

As with shoot formation, elongation was also promoted by inclusion of BA, although the differences in shoot height between the treatments were not significant after 28 d. The number of shoots per productive explant was significantly increased by inclusion of BA after 28 and 56 d (Table 1) and shoot number was found to increase with increasing BA concentration. Shoots produced on media containing a higher concentration of BA (0.8 and 1.0 mg l^{-1}) after 56 d appeared glassy, typical of hyperhydric malformation, and possessed brittle twisted leaves. Higher concentrations of BA (0.8 and 1.0 mg l^{-1}) also stimulated the growth of callus at the cut surfaces of explants.

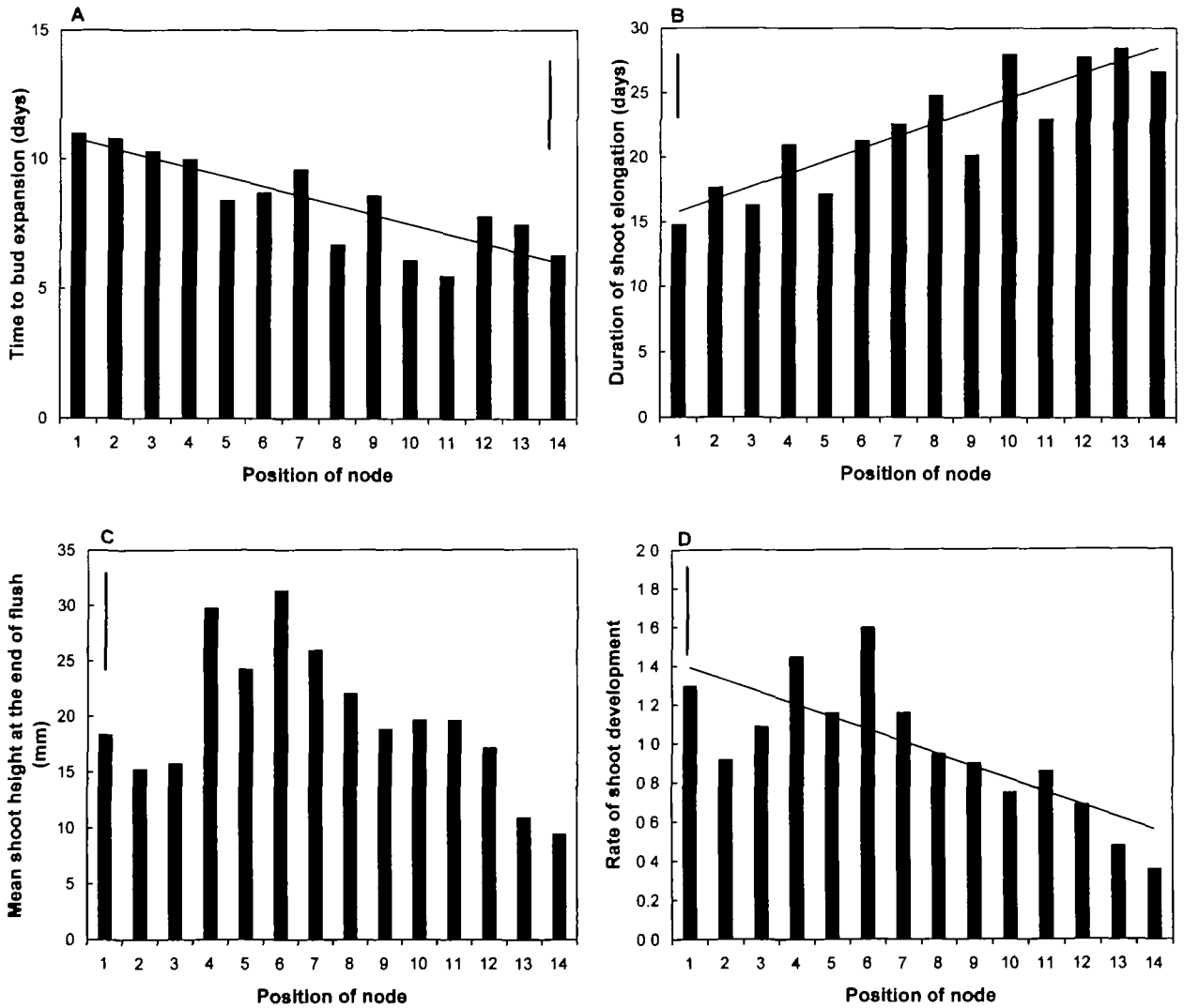

Fig. 1. The influence of explant position on axillary shoot development in nodal cultures of oak. (A) Time from culture initiation to the start of axillary bud expansion (bar represents sed(df)=3.29(73) ns, estimated slope of linear trend=0.4±0.13**). (B) Duration of axillary shoot elongation (bar represents sed(df)=4.73(73)**, estimated slope of linear trend=0.95±0.185***). (C) Axillary shoot height at the end of elongation growth flush (bar represents sed(df)=8.42(73) ns). (D) Rate of shoot development (shoot height/duration of shoot elongation) (bar represents sed(df)=0.41(73)*, estimated slope of linear trend=−0.6±0.016***). Position numbers refer to the number of axillary buds from the root collar to the apex, all shoots used possessed 14 nodes. Values are means with $n=10$. Asterisks denote significant differences with $*P<0.05$, $**P<0.01$ and $***P<0.001$. ns=differences not significant.

Greater numbers of multiplication segments were excised when BA was included in the basal medium, although differences between treatments were only significant after 56 d culture. However, after each subculture there was a significant trend of increasing segment number with BA concentration (Table 1). There were significant differences in multiplication rates between treatments after each subculture (Table 1), with explants treated with 1.0 mg l^{-1} BA having the greatest multiplication coefficient after 56 d. Much of the variation in multiplication coefficients was attributable to differences in the number of explants forming axillary shoots (Table 1).

Influence of explant source: The number of explants forming axillary shoots differed significantly between individual seedlings but differences between provenances were not significant (Table 2). The former position of nodal explants in the seedling shoot significantly influenced the formation of axillary shoots *in vitro*. Axillary shoot formation was highest in nodes formerly associated with a leaf (positions 8–11), except in explants excised from the apex which contained multiple buds (Table 2). Axillary shoot formation was reduced in the leafless nodal explants excised from the base of seedling shoots (positions 1–3), these explants also contained multiple axillary buds and many remained quiescent.

Axillary buds in explants excised from apical regions (positions 9–14) began shoot development 5–8 d after initiation. The time taken for buds to commence shoot growth increased significantly ($P<0.01$) as explants were taken from more basal positions (Fig. 1A). Although axillary buds took longer to commence development from basal explants, the duration of shoot growth from these explants was relatively short. Typically axillary shoots from basal explants ceased elongation growth within 20 d whereas those from apical explants required around 25 d or more to complete elongation growth (Fig. 1B). The duration of axillary shoot elongation increased significantly ($P<0.001$) as explants were excised from more apical positions. Greater durations of shoot elongation did not lead to the formation of taller shoots. In fact, the tallest shoots were formed by axillary buds on explants in positions 4–7 (Fig. 1C), buds on these explants were formerly leafless and produced shoots whose elongation growth was relatively rapid. Differences between positions in the heights of the shoots at the end of elongation growth were not significant. As a consequence of the greater durations of shoot elongation the rate of development of axillary shoots from apical explants was significantly slower compared to that from basal explants (Fig. 1D). *In vitro* development of axillary shoots followed a sigmoidal pattern (Fig. 2).

There were significant differences in the number of explants that produced multiple axillary shoots (Table 3). Explants excised from apical and basal regions of seedlings (positions 1–4 and 12–14, respectively) tended to produce only a single axillary shoot *in vitro*. Multiple shoot formation occurred in explants excised from mid-stem positions (5–11). The development of multiple shoots was not synchronous with the formation of the initial axillary shoot, which develop from the isolated bud on each explant. Multiple shoots arose from lateral buds formed at the base of the axillary shoot, their development began once elongation of the initial axillary shoot was nearing completion (Fig. 3). There were significant ($P<0.001$) acropetal trends in the time taken for multiple shoot formation to commence and for completion of axillary shoot growth. Basal explants formed multiple shoots more quickly than apical explants (Figs 2, 3),

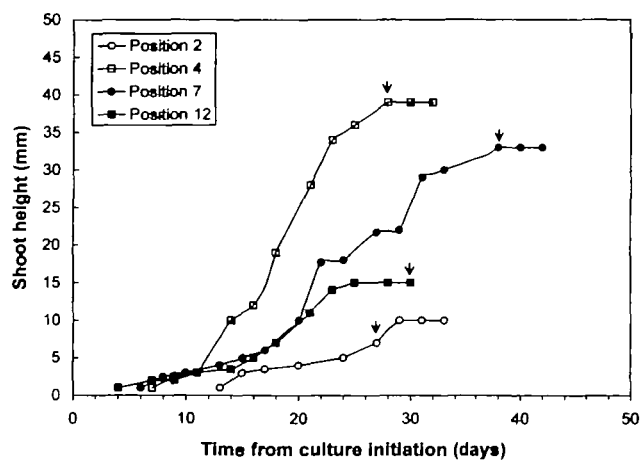

Fig. 2. Examples of the pattern of shoot development from nodal explants excised from different positions in shoots of *Q. robur*. Position numbers refer to the number of axillary buds from the root collar to the apex, all shoots used possessed 14 nodes. Arrows indicate the time at which shoot formation from basal lateral buds commenced.

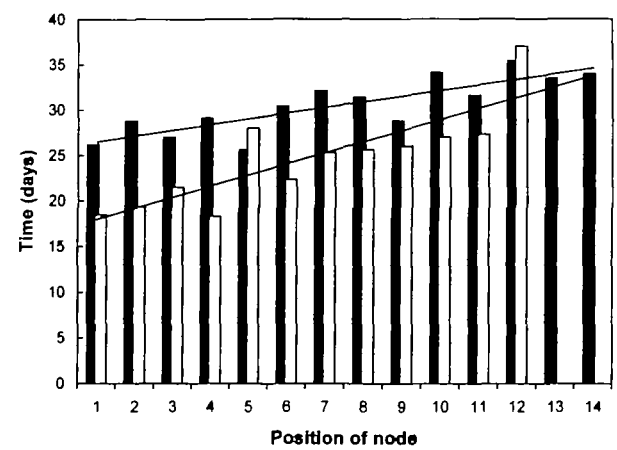

Fig. 3. The influence of explant position on the time from initiation to the completion of axillary shoot elongation (■, estimated slope of linear trend = 0.6 ± 0.18***) and the time to the start of multiple shoot development (□, estimated slope of linear trend = 1.2 ± 0.34***). Position numbers refer to the number of axillary buds from the root collar to the apex, all shoots used possessed 14 nodes.

Table 4. *The effect of temperature on axillary shoot formation in nodal explants of* Q. robur

Values are based on 24 explants per treatment.

Temperature (°C)	Number of productive explants forming a shoot	Mean number of shoots per explant	Mean length of tallest shoot (mm)	Mean number of segments per productive explant	Multiplication coefficient (mean number of segments per explant)
15	11	1.0	9.5	1.1	0.5
20	23	1.2	21.9	2.5	2.4
25	19	2 0	23.1	3.4	2.7
30	16	1.9	14.1	2 5	1.6
Significance χ^2(df)	17.5(3)***				
sed(df)		0.46(65)**	4.18(65)***	0 57(65)**	0.47(92)***

Asterisks denote significant differences; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

reflecting the shorter period of time required for the primary axillary shoots from basal explants to complete elongation growth. There were no differences in the number of additional shoots formed by explants from different positions.

Influence of culture environment: There were significant differences between temperature treatments in the number of explants with shoots ($P<0.001$) and the number of new segments per explant ($P<0.001$). Explants cultured at 15 °C produced the poorest responses (Table 4) with most remaining quiescent. After the comparison of axillary shoot formation between explants incubated at 20 °C and 25 °C and those incubated at 15 °C and 30 °C ($\chi^2(df)=12.1(1)$, $P<0.001$) had been made, the residual tretment differences were not significant. Shoot elongation was promoted by increasing temperature resulting in greater numbers of new segments being produced. However, this latter trend was not linear and at high temperature (30 °C) shoot elongation, and thus segment number, was reduced (Table 4). Incubation of explants at 30 °C promoted callusing of their cut surfaces, such that callus engulfed axillary buds and thus appeared to restrict their development. High temperatures also influenced axillary shoot development producing shoots with brittle stems and twisted leaves. Multiplication coefficients were highest for explants maintained at 20 °C and 25 °C. Although axillary shoot formation was greater amongst explants incubated at 20 °C than at 25 °C, similar multiplication rates resulted because shoot proliferation and elongation was promoted amongst the responsive explants at the higher temperature (Table 4).

The principal effect of daylength treatments was on the number of explants forming axillary shoots. After the comparison of daylengths of 16 h or more with shorter daylengths ($\chi^2(df)=9.5(1)$, $P<0.01$) had been made, the residual treatment differences were insignificant. At daylengths of less than 16 h the majority of explants remained quiescent. In each of the treatments for those explants which produced shoot growth, there were no significant differences in the number of shoots formed, the length of the tallest shoot or the number of new stem segments produced (Table 5). Although multiplication coefficients increased with increasing daylength, reflecting the greater numbers of explants forming axillary shoots under longer days, neither this trend nor differences between treatments were significant.

Table 5. *The effect of daylength on axillary shoot formation in nodal explants of* Q. robur

Values are based on 24 explants per treatment

Daylength (h)	Number of productive explants forming a shoot	Mean number of shoots per explant	Mean length of tallest shoot (mm)	Mean number of segments per productive explant	Multiplication coefficient (mean number of segments per explant)
8	9	1.4	18.3	2.4	0.9
12	8	1.9	23.6	3.5	1.2
16	15	1.6	18.0	2.6	1.6
24	17	1.5	20.2	2 7	1.9
Significance χ^2(df)	10.01(3)*				
sed(df)		0.48(45)	4.35(45)	0.81(45)	0.54(92)

Asterisks denote significant differences; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Table 6. *The effect of temperature with daylength on the number of nodal explants of* Q. robur *forming an axillary shoot,* n = *30*

Daylength (h)	Temperature (°C)	
	15	20
12	5	12
16	11	18
Significance		
Deviance change for effect of daylength	$\chi^2 = 5.1$, df = 1, $P < 0.05$	
Deviance change for effect of temperature	$\chi^2 = 7.3$, df = 1, $P < 0.01$	
Deviance change for effect of interaction of daylength with temperature	$\chi^2 = 0.1$, df = 1, ns	

Increasing incubation temperature and daylength both significantly promoted formation of axillary shoots from nodal explants (Table 6). There was no significant interaction of temperature with daylength on the number of explants forming shoots under the regimes investigated.

Culture establishment

Influence of BA: Callus formation was promoted at high BA concentrations (0.8 and 1.0 mg l^{-1} BA) with stem segment explants becoming engulfed in callus tissue such that the origin of shoots was masked. At lower BA concentrations callus formation was confined to the cut basal surface of the explant. Inclusion of BA in the culture medium significantly promoted axillary shoot development (Table 7), after the comparison of BA with no BA (χ^2(df) = 14.8(1), $P < 0.001$) had been made, the residual treatment differences (χ^2(df) = 0.52(5)) were not significant. Increasing BA concentrations tended to promote the number of shoots and new stem segments formed per explant and to increase multiplication coefficients (Table 7). Although analyses of these data revealed significant linear trends, deviations from these trends were also significant. The principal effect was found to be the incorporation of BA in the culture medium, which significantly promoted shoot number ($P < 0.001$), shoot height ($P < 0.01$), segment number ($P < 0.001$) and multiplication rates ($P < 0.001$). There was little to choose between the BA treatments in terms of multiplication coefficients, the best response was obtained at 0.4 mg l^{-1} BA.

Influence of incubation temperature: Shoot formation was promoted at the higher incubation temperatures of 25 °C and 30 °C (Table 8), the number of explants with axillary shoots was reduced at incubation temperature of 20 °C after 28 d (χ^2(df) = 5.76(1), $P < 0.05$) and 56 d (χ^2(df) = 7.36(1), $P < 0.01$). Higher incubation temperatures also promoted shoot elongation and the number of shoots and segments formed, although none of these differences were significant. The responses of explants were improved after subculture. Significantly greater rates of multiplication were obtained from explants incubated at higher temperatures after 28 and 56 d due to the increase in the numbers of explants forming shoots at these temperatures.

Discussion

The effectiveness of BA treatments for the initiation of shoot growth from nodal explants of *Q. robur* has been confirmed by other workers (Chalupa, 1984*b*; Favre and Juncker, 1987; San-José *et al.*, 1988, 1990). These results, in common with those of Evers *et al.* (1988), indicate that the effect of BA is through the activation of axillary shoot development. Vieitez *et al.* (1985) reported that the

Table 7. *The effect of BA on the establishment of shoot growth in nodal stem segments of axillary shoots of* Q. robur *formed* in vitro

Values are based on 30 explants per treatment.

Concentration of BA (mg l^{-1})	Number of productive explants forming a shoot	Mean number of shoots per explant	Mean length of tallest shoot (mm)	Mean number of segments per productive explant	Multiplication coefficient (mean number of segments per explant)
0	12	1.2	9.6	1.2	0.5
0.1	23	1.9	16.4	3.2	2.5
0.2	22	1.3	16.7	2.1	1.6
0.4	24	2.3	16.0	3.6	2.8
0.6	22	2.4	16.1	3.3	2.4
0.8	23	2.4	14.4	2.9	2.3
1.0	23	2.5	15.9	3.6	2.8
Significance					
χ^2(df)	15.3(6)***				
sed(df)		0.38(142)***	2.47(142)	0.66(142)**	0.52(203)***
Lin(SE)[a]		—	0.9(1.69)	—	—

Asterisks denote significant differences; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
[a]Lin estimated value for slope of linear trend. No values are presented where deviations from the linear trend are significant.
SE = standard error.

Table 8. *The effect of temperature on the establishment of shoot growth in nodal stem segments of axillary shoots of* Q. robur *formed* in vitro

Values are based on 20 explants per treatment.

Temperature (°C)	Number of productive explants forming a shoot	Mean Number of shoots per explant	Mean length of tallest shoot (mm)	Mean number of segments per productive explant	Multiplication coefficient (mean number of segments per explant)
28 d					
20	7	1.3	12 0	1.9	0.7
25	14	2.2	15.5	3.1	2.2
30	13	2.0	11.2	2.3	1.5
Significance					
χ^2(df)	5.8(2)				
sed(df)		0.54(31)	2.68(31)	0.60(31)	0.48(57)*
56 d					
20	9	1.6	15.0	2.6	1.2
25	16	2.0	18 5	3.6	2.9
30	16	2 4	16.3	3.6	2.9
Significance					
χ^2(df)	7.4(2)*				
sed(df)		0.50(38)	2.97(38)	0.72(38)	0.63(57)**

Asterisks denote significant differences; $*P<0.05$, $**P<0.01$

use of BA at 1.0 mg l^{-1} was only effective in initiating growth from shoot tip explants whereas mid-stem nodal explants just callused. It was found that increasing the concentration of BA promoted callus growth, but nodal explants did produce axillary shoots, although their morphology was affected. The formation of abnormal shoots in response to high BA concentrations has been observed previously in oak (Favre and Juncker, 1987) and other woody species (Barghchi, 1988). In these studies an incubation temperature of 22 °C was used, slightly lower than that reported by Vieitez *et al.* (1985). It was found that both callusing and abnormal shoot morphologies also resulted when explants were incubated at higher temperatures and may explain why Vieitez *et al.* (1985) were previously unable to obtain shoots from nodal explants. The combination of high BA concentrations and incubation temperatures above 25 °C is less suitable for the initiation of oak cultures.

Explants produced a single growth flush in response to BA inclusion in the medium. Repeated flushing of oak shoots has been obtained on culture medium supplemented with activated charcoal (Favre and Juncker, 1987). In this work transfer of shoots to fresh medium did not lead to further flushes of shoot growth. Further shoot development was obtained from leafless buds at the base of axillary shoots. Reducing the level of apical dominance, by increasing the concentration of BA and the transfer of nodal explants to fresh medium following the excision of axillary shoots, increased shoot formation. The numbers of shoots formed in response to BA treatments were consistent with those reported by Chalupa (1988). For initiation of explants under an incubation temperature of 22 °C the most effective BA concentration was 1.0 mg l^{-1}. Although the frequency of axillary shoot formation tended to be lower in nodes treated at this concentration, this was more than compensated for by the stimulation of multiple shoot formation which presented higher numbers of stem segments for shoot multiplication.

A variety of incubation conditions have been used in previous studies on *in vitro* growth of oak. Shoot formation was found to be strongly influenced by temperature. Increasing incubation temperature promoted shoot formation although high temperatures were not beneficial. The promotion of callus growth in explants at 30 °C caused a decrease in shoot formation similar to the problems with callogenesis encountered by Favre and Juncker (1987). Callogenesis and abnormal shoot development are reduced at lower incubation temperatures (20–22 °C), suggesting that the high temperatures employed in previous studies are not optimal for culture initiation. The stimulation of shoot elongation at temperatures of 20 °C and 25 °C is comparable to natural growth responses to temperature observed by Farmer (1975).

Long days are known to stimulate flushing of oak shoots (Longman and Coutts, 1974; Harmer, 1989) and the frequency of shoot formation *in vitro* was improved by increasing daylength. Low temperature (15 °C) and short daylengths (12 h or less) were not suitable for the initiation of axillary shoot growth *in vitro*. Under long days it remains unclear whether it is the period of light or amount of light energy received in a given irradiance

period which is important. The observations that high light intensities favour shoot elongation in *Q. rubra* (Farmer, 1975) and that low light intensities favour quiescence suggest that it is the amount of light energy received which is important. Additionally, the emergence of oak buds from winter dormancy is regulated by temperature and not increasing daylength. It has also been shown that light intensity is important in the growth of shoots of other woody plants in culture (Pierik, 1987; Evers, 1987).

In addition to light period and irradiance levels the quality of light in the culture environment is known to influence responses (Pierik, 1987). Red light increases axillary bud activity and is known to promote shoot proliferation in culture (Read *et al.*, 1979, 1988). Most culture rooms are illuminated by fluorescent tubes, usually Warm white or Gro-lux types which give out a high proportion of orange-red light and usually provide the best results (Pierik, 1987; Schneider-Moldrickx, 1983). Such environments have proved adequate for the culture of oak tissues but the importance of light quality and particularly the role of red light in the activation of axillary shoot growth have not been investigated.

No difference was found in the overall performance of explants excised from seedlings of either provenance used. In common with other reports (San-Jose *et al.*, 1990), large differences in the response of explants from individual seedlings were observed. Genotypic differences caused some variation in the response of explants between experiments with similar treatments, particularly in terms of the number of explants forming axillary shoots. The present results have shown that these differences can be minimized by excising explants from mid-stem positions, regions where seedling internode elongation is maximal.

Topophysical effects, whereby the position of the explant in the plant influences the growth in culture, have been noted in several species. Pierik (1987) cites several examples ranging from the position of tissues excised from bulbs to gradients of regeneration in tobacco shoots. In tree species, Evers (1987) reports that responses are improved in Douglas fir cultures when explants are taken from lower branches. The effect of explant position within a single oak shoot has been studied by Volkaert *et al.* (1990). When explants were excised during shoot elongation, development of axillary shoots was superior in basal explants, presumably as a consequence of the advanced development of these nodes which complete internode elongation before more apical internodes. In this respect, both positional and developmental differences in the lignification of explants can be envisaged, a factor previously noted by Favre and Juncker (1987) to be an important determinant of shoot formation in nodal explants of oak.

In the present study, explants from basal and apical positions contained more than one axillary bud. Poor responses were obtained from buds at the apex of seedling shoots in agreement with Volkaert *et al.* (1990) who used similar explants. In contrast to the study by Volkaert *et al.* (1990), the present results suggest that basal explants responded poorly. However, in this study the response of individual nodes was followed and not the explant as a whole. In these explants, the first bud to develop appeared to become dominant thereby restricting the development of the remaining buds.

Bud size and state of development are known to be determinants of branching in *Q. petraea* (Harmer, 1991), with shoots formed predominantly from large buds at the shoot apex or whorl. Such buds are usually found in leaf axils and defoliation, or partial removal of leaves, stimulates the formation of branches in oak (Hilton *et al.*, 1987). Small leafless buds at the base of shoots do not normally form branches *in vivo* due to their size and rudimentary development (Harmer, 1991). The state of bud development is known to influence culture initiation. Favre and Juncker (1987) observed that the number of leaves formed by axillary shoots of oak corresponds to the number of leaf primordia contained in the original bud. The present results show that a high proportion of the well-developed larger buds excised from apical and mid-stem positions initiate axillary shoot growth rapidly, although completion of shoot development requires a relatively long time. In contrast, fewer of the small leafless buds from basal explants develop axillary shoots. Where shoot development takes place from basal explants, growth starts later, but shoots develop quickly reflecting the rudimentary nature of these buds. Clearly, the responses observed during culture initiation reflect patterns of growth predetermined on the source plant.

The episodic nature of oak shoot growth is retained in culture (Favre and Juncker, 1987; Volkaert *et al.*, 1990) suggesting that growth remains dominated by endogenous controls and is typical of species where growth occurs by extension of preformed buds (McCown and McCown, 1987). The response of explants from different positions on seedling shoots supports this view. Multiple shoots were formed from lateral buds at the base of axillary shoots but only after completion of axillary shoot elongation. The formation of these shoots, despite the fact that explants were not transferred to fresh medium, suggests that cultural conditions were not limiting to growth at the time when elongation of the axillary shoot ceased.

Continued growth of seedling tissue was obtained by culturing sections from axillary shoots. As with nodal explants at initiation, shoot growth from stem sections was increased by the presence of BA. In previous studies, BA concentrations were reduced from 1.0 to 0.1 mg l^{-1} to avoid the adverse effects of prolonged exposure to high concentrations (Vieitez *et al.*, 1985; San-José *et al.*, 1988; Chalupa, 1988), these workers used incubation temperatures between 24 °C and 30 °C. In the present study,

productivity of stem sections was better at a higher BA concentration (0.4 mg l^{-1}) but a lower incubation temperature (22 °C) was used. It was found that productivity of explants was influenced by temperature but, as with nodal explants at initiation, higher temperatures promoted shoot formation but also stimulated callus growth.

Shoot development took longer from buds possessed by apical explants, requiring more than the 28 d used as a subculture interval. Developmental differences will result between apical and nodal stem sections cut from shoots still undergoing elongation, as basal internodes complete elongation before more apical internodes. It is known that explants excised from shoots still undergoing elongation perform poorly and the same is thought to apply to stem segments cut from *in vitro*-cultured shoots (Volkaert *et al.*, 1988). Developmental differences can be minimized by excising stem segments after completion of shoot elongation. The time required to complete elongation depends upon the explant source, only explants taken from mid-stem and basal positions complete elongation growth within the usual 28 d subculture interval.

In summary, treatments which promoted shoot development from nodal explants of *Q. robur* and overcame quiescence of tissues were low concentrations of BA and daylengths of 16 h or more with an incubation temperature of 20 °C or 22 °C. Higher incubation temperatures (25 °C) reduced the number of active explants but promoted multiple shoot formation from those explants which responded and were therefore desirable for shoot multiplication. BA was necessary for continued shoot growth from subcultured stem sections. Reactivity of explants was maintained by culturing at 22–25 °C and shoot multiplication was improved by culturing at 25 °C and by subculturing stem segments.

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